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A dominant antigen is necessary but not sufficient for effective neoantigen vaccination in murine mesothelioma

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Abstract

Neoantigen vaccines have emerged as a promising strategy for personalised cancer treatment, however the features of neoantigen vaccines that confer effective tumour protection remain poorly defined. Further, their application for low tumour mutation burden (TMB) cancers has been largely unexplored. We hypothesised that vaccines targeting spontaneously immunogenic neoantigens would provide superior protection to neoantigens that induce *de novo* T-cell responses post vaccination. We also hypothesised that increasing the breadth of neoantigen targets or including a dominant antigen would enhance vaccine efficacy. In a mouse model of the low TMB cancer mesothelioma, only vaccines that combine a dominant antigen, spontaneously recognised antigens, and neoantigens with vaccine-inducible T-cell responses conferred protection, however immunoediting of only the dominant antigen was sufficient for vaccine failure. This study highlights potential antigen dominance hierarchies that can influence vaccine efficacy and demonstrates the feasibility of neoantigen vaccines in mesothelioma, with potential implications for other low TMB tumours.

Introduction

Tumour-specific T-cell responses underlie the success of immune checkpoint inhibitor (ICI) therapy, which is now used in the treatment of many cancer types[1]. Despite its widespread use most patients do not respond to ICI and can experience severe immune-related adverse events as a result of non-specific T-cell activation. Newer, more targeted approaches that employ tumour-specific T-cell responses, such as personalised neoantigen vaccines, are emerging as an alternative or adjunct to ICI therapy[2,3]. Neoantigen vaccines target somatic tumour mutations, aiming to induce strong tumour-specific T-cell responses while minimising off-target effects. Across a range of cancers, pre-clinical mouse studies[4-6] and multiple clinical trials[2,7-12] have demonstrated that neoantigen vaccines are safe and induce neoantigen-specific immune responses. Importantly, the first randomised trial to demonstrate a clinically meaningful benefit of neoantigen vaccination combined with anti-PD1 over anti-PD1 alone was recently published[11].

Despite the promise of neoantigen vaccines, selecting the most effective targets remains a key challenge for their routine clinical application. This is particularly important for tumours with a low to moderate tumour mutational burden (TMB), which represent the majority of cancer types[13]. In contrast to high TMB cancers like melanoma, tumours with a low to moderate TMB have fewer candidate neoantigens available for inclusion in vaccines. While computational prediction can identify large numbers of candidate neoantigens, only a small proportion appear to be truly immunogenic regardless of tumour type[2].

Of those that are immunogenic, neoantigens can be classified as either “spontaneous” or “induced”: spontaneous neoantigens are those recognised naturally by T-cells during tumour growth, whereas induced neoantigens are those that generate *de novo* T-cell responses only by

vaccination. Spontaneous T-cell responses are typically detected against only 2–5% of predicted neoantigens in tumour-bearing patients, whereas vaccination can induce *de novo* responses to as many as 40–60% of vaccine candidates[3]. Very few studies pre-screen for immunogenicity[14-17], and whether spontaneous neoantigens are more effective vaccine targets than induced neoantigens remains unknown. If spontaneous neoantigens are superior, this would support pre-screening candidates for immunogenicity before vaccine manufacture, despite the additional time and complexity involved. However, neither class alone may be sufficient to generate an effective antitumour response, and vaccine efficacy may instead depend on the combined breadth and composition of the antigenic response. Further, tumour-intrinsic properties such as neoantigen expression, immunodominance, and intratumoural heterogeneity may further influence efficacy by determining the proportion of tumour cells susceptible to vaccine-induced immunity[18].

To address these questions we employed a mouse model of a low TMB tumour, mesothelioma; a rare, aggressive, asbestos-associated cancer with limited treatment options and a poor prognosis[19-21]. Several spontaneously immunogenic neoantigens have been defined previously in this mouse model[22-25]. We first identified additional spontaneous neoantigens, as well as multiple induced neoantigens, derived from somatic mutations in this model. We examined the protective efficacy of these two classes of antigen, alone and in multivalent formulations, and assessed whether efficacy was enhanced by including a dominant nominal antigen. Targeting either spontaneous or vaccine-induced T-cell responses failed to protect mice from tumour challenge, even when up to ten bona fide neoantigens were combined. By contrast, adding a single strongly immunogenic nominal antigen conferred significant protection. Protection remained incomplete, however, and tumour outgrowth in non-responding mice was associated with immunoediting and loss of the dominant antigen.

Results

Identification of mesothelioma neoantigens that elicit spontaneous or vaccine-induced T-cell responses

In our previous studies using the AB1-HA mouse mesothelioma model, thirty highly expressed neoantigens with strong predicted MHC-I binding affinity were tested for immunogenicity[24,25]. Two of these neoantigens, derived from non-synonymous single nucleotide variants (nsSNV) in the genes unc-45 myosin chaperone A (Unc45a)[24] and ubiquinol-cytochrome c reductase core protein 2 (Uqcrc2)[24], were spontaneously recognised by T-cells in tumour bearing mice. Here we first sought to identify spontaneous neoantigens additional to Unc45a and Uqcrc2 in this model. Neoantigen peptides were predicted using an updated version of our previously described bioinformatics pipeline[24] that prioritised an MHC-I IC50 (<500 nM) consensus score using Antigen Garnish[26] and a differential agretopicity index (DAI)[27] >4 (Figure 1A). Antigen Garnish also predicted MHC-II peptides, and the top 20 that also contained an MHC-I epitope were tested for spontaneous immunogenicity (Supplementary Table 1). A total of 151 peptides corresponding to mutations in 105 genes were tested in pools of up to 12 peptides per pool. Four peptides were derived from indels (insertions-deletions), and the rest were derived from nsSNV.

To evaluate the spontaneous immunogenicity of candidate neoantigens, IFNγ responses were assessed using tumour-draining lymph node (LN)- and spleen-derived T-cells from AB1-HA tumour-bearing mice. T-cell responses were detected to peptide pools 3, 8, 20, and 24 (Figure 1B). Deconvolution of these pools revealed spontaneous, albeit weak, T-cell responses to two novel SNV-derived neoantigens. One neoantigen was derived from the vomeronasal 2 receptor 67 (Vmn2r67) gene and was due to 1198G>C SNV that resulted in a A400P amino acid substitution. The other novel neoantigen was derived from a 407A>T SNV in the elongator

complex protein 2 (Elp2) gene and led to a D136V amino acid substitution. To confirm the minimal $CD8^+$ T-cell epitopes from the four spontaneous neoantigens, mice were vaccinated with corresponding synthetic long peptides (SLPs) and responses to the predicted minimal $CD8^+$ epitopes were subsequently assessed. $CD8^+$ epitopes were re-confirmed for Unc45a[25] (IYEVVRSLV, Figure 1C) and Uqcrc2[24] (SYMAPSTVL, Figure 1D). A $CD8^+$ epitope was confirmed for Vmn2r67 (VPHSLHEML, Figure 1E) but not Elp2 (Figure 1F). Further characterisation of the Elp2-specific response demonstrated no recognition of the predicted minimal epitope, whereas the SLP appeared to elicit a predominantly $CD4^+$ T-cell response (Figure 1G). In total only four of the 105 gene mutations tested, Unc45a, Uqcrc2, Elp2 and Vmn2r67 elicited T-cell responses in tumour bearing animals, corresponding to a spontaneous immunogenicity of 3.8% (4/105).

Given that mutation position can influence peptide immunogenicity, we explored the impact of each mutation on the predicted peptide-MHC-I and T-cell receptor (TCR) interactions. Unc45a and Uqcrc2 were predicted to bind H2-K^d, with the mutations altering putative TCR contact residues at P7 and P4 respectively (Figure 1H). In contrast, Vmn2r67 was predicted to bind H2-L^d, with the A400P amino acid substitution introducing a preferred proline at the P2 MHC-I anchor residue (Figure 1I). The introduction of a novel anchor residue is consistent with the elevated DAI score of this peptide (DAI = 4.02; Supplementary Table 1).

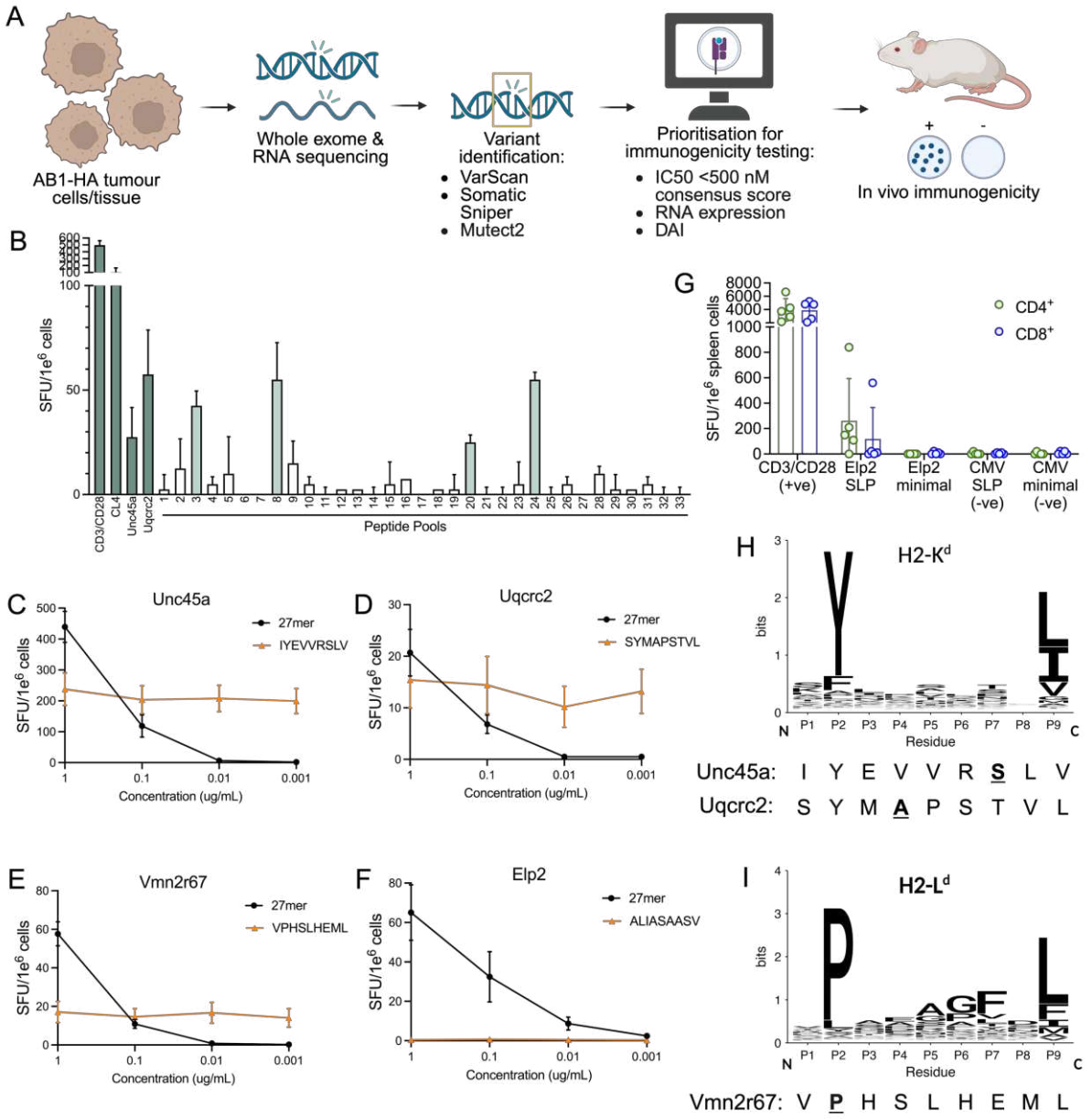


Figure 1. Spontaneously recognised neoantigens can be identified in in a model of mesothelioma. (A) Schematic of bioinformatics pipeline used for genomic prediction and testing of neoantigens. Tumours and matched normal tissue underwent whole exome and RNA sequencing, and non-synonymous single nucleotide variants and small indels were called using VarScan, Somatic Sniper, and Mutect2. Neoantigens were predicted and prioritised for immunogenicity testing based on an IC50 (<500 mM) consensus score, expression of the mutation, and a high differential agretopicity index (DAI). Candidate neoantigens were ranked and the top 151 peptides were tested for immunogenicity in tumour-bearing mice. **(B)** Screening of T-cell responses to pools of predicted neoantigen peptides. An IFNγ response was observed in pools 3, 8, 20, and 24. **(C-F)** Minimal epitope mapping for Unc45a, Uqcrc2,

Vmn2r67, and Elp2. Mice (n=5 per group) were vaccinated with a 27mer SLP corresponding to each neoantigen and poly I:C. Spleens were used to test IFNγ response to minimal epitopes at 1 ug/mL to 0.001 ug/mL. **(G)** $CD4^+$ vs $CD8^+$ IFNγ response to long and minimal Elp2 peptides from Elp2-vaccinated spleens. **(H-I)** Sequence logo plots for the BALB/c MHC I alleles to which the 9 mer spontaneous neoantigen peptides are predicted to bind. ELISpot data are mean ± SD spot forming units per million cells.

Most neoantigen vaccine trials do not pre-screen for immunogenicity of each vaccine candidate but regularly report T-cell responses to 40-60% of epitopes following vaccination[7-9,28,29]. We tested whether this rate of vaccine-inducible immunogenicity was also true in our model. Such neoantigens are herein referred to as "induced" neoantigens. We assessed the inducible immunogenicity of thirty predicted neoantigens by vaccinating mice with pools of 5 SLPs emulsified in Montanide. IFNγ responses were detected on day 14, from draining LN derived T-cells, to 63% (19/30) of the SLP (Figure 2A, Table 1). For 25 of these SLP, MHC-I epitopes could be predicted and of these 32% (8/25) induced detectable T-cell responses (Figure 2B). To validate these findings, we tested the immunogenicity of an independent set of 25 predicted neoantigens employing a different adjuvant, poly I:C, which is frequently used in clinical trials[7,9,28,30]. T-cell responses were detected to 52% (13/25) of SLPs and to 24% (6/25) of corresponding predicted MHC-I epitopes (Figure 2C & 2D, Table 1). Taken together, T-cell responses were detectable to 58% (32/55) of the SLPs tested, with relative immunogenicity similar across adjuvants.

Table 1. Genomically predicted neoantigens tested for inducible immunogenicity. Mutations are indicated as underlined and bold letters. Binding affinity scores are from NetMHCPan4.0 for the predicted minimal epitope (pMHC-I). EL rank binding categories: SB = strong binder (≤0.5); WB = weak binder (>0.5-2.0).

Gene	SLP		pMHC-I		IC50(nM)	%EL rank
	Sequence	Immunogenic	Sequence	Immunogenic		
Anapc2	LLFWAYIQAMLTN**V**ESLSLERIYSMLR	Yes	AYIQAMLTN**V**	Yes	46	0.11 (SB)
Ap3d1	PVPFQLPPGVSNE**P**QFVFTIQSIVMAQ	Yes	VSNE**P**QFVF	No	8785	0.22 (SB)
Arhgap5	INLASMEVTVNAV**S**GALKAFFADLPDP	No	V**S**GALKAFF	No	6136	0.70 (WB)
Brwd3	IQSQKRRRPRYRK**L**LRSSSSSLSSSRA	No	RYRK**L**LRSSSSSL	No	950	1.30 (WB)
Cog7	DYLINVMDALGLQ**A**SRTLQNIAALLKA	Yes	LGLQ**A**SRTL	No	7647	0.59 (WB)
Dpp9	ESEAQRQAAARAV**T**KNVQPFVIYEEVT	No	RAV**T**KNVQPF	No	13882	2.41
Dst	DTLPREKGRMRFH**S**LQNVQIALDYLKR	Yes	KGRMRFH**S**L	N/A	1992	0.08 (SB)
Exosc5	GSLLACCLNAACM**P**LVDAGVPMRALFC	No	LNAACM**P**L	No	15570	0.29 (SB)
Ganab	VRWYQMGAYQPFF**Q**AHAHLDTGRREPW	Yes	QPFF**Q**AHAHL	Yes	782	0.22 (SB)
Glud1	LRIIKPCNHVLSL**F**FPIRRDDGSWEVI	No	KPCNHVLSL**F**F	No	1725	1.93 (WB)
Hjurp	KYLTQVDILPQDE**G**YFKNAEKRGGKDT	Yes	LPQDE**G**YF	No	9400	1.01 (WB)
Lmf1	FLFLGRRMRILHG**G**LQILFQVILIISG	Yes	RMRILHG**G**LQI	No	11052	10.61
Mdn1	TGCGKTTVCQMFS**V**LANQKLYSVNCHL	No	FS**V**LANQKL	N/A	16022	1.70
Mdn1 (alt)	LQTNITDDFVIPL**G**PRWNMQALDIIRN	Yes	IPL**G**PRWNM	N/A	70	0.01 (SB)
Mon1a	EALRTPYYSVAQV**A**IPDLRHFLYKSKS	Yes	YYSVAQV**A**I	No	49	0.02 (SB)
Mrpl23	HKNVRIKKPDYKV**G**YVQLAHGQTFTFP	Yes	**G**YVQLAHGQTF	No	2951	0.84 (WB)
Mrpl28	QAGAAEYYRAYLE**G**ECVEWLLRYLELG	No	AYLE**G**ECVEWL	No	1013	0.73 (WB)
Nup160	PSVIAIAGSSSAK**K**MSALLVQAGLFDT	Yes	SSAK**K**MSAL	N/A	6047	0.22 (SB)
Pop7	AELDPVEYTLRKR**R**PHRLPRRPNDIYV	No	YTLRKR**R**PHRL	No	18909	5.50
Rdx	TQQDVKKENPLQF**N**FRAKFFPEDVSEE	Yes	NPLQF**N**FRAKF	Yes	2603	0.69 (WB)
Rpn1	YILFFTVIIYVRL**E**FSITKDPAAEARM	No	IYVRL**E**FSI	No	49.9	0.09 (SB)
Sf3b2	EEEIDRTPWGELE**L**SDEESSEEEEEEE	No	TPWGELE**L**	No	2734	0.32 (SB)
Ssx2ip	PACTSKLTKSLPA**L**PSTSDFRQTHSCV	No	LPA**L**PSTSDF	N/A	2597	0.21 (SB)
Tmem161a	VTVVSLQYLTPLI**F**TLHCTLLLKTLGG	No	TPLI**F**TLHCTL	No	785	1.09

Gene	SLP		pMHC-I		IC50(nM)	%EL rank
	Sequence	Immunogenic	Sequence	Immunogenic		
Trmt5	DGKDFIQGPVREE**V**MLRLGLSAEAKPS	Yes	QGPVREE**V**ML	Yes	2723	0.47(SB)
Ube2b	GSICLDILQNRWS**A**TYDVSSILTSIQS	Yes	RWS**A**TYDVSSI	No	2814	4.93
Unc45a	MTFPGERIYEVVR**S**LVSLLHLSCSGLQ	Yes	IYEVVR**S**LV	Yes	21	0.37 (SB)
Zfp524	DGPQGSGSKRAPH**V**CPVCLRAFPYLSD	Yes	APH**V**CPVCL	Yes	309	0.16 (SB)
Zfp768	ERPYRCAVCGKGF**F**RSSTLLQHHRVHS	Yes	KGF**F**RSSTL	Yes	637	0.03 (SB)
Uqcrc2	EIGSQALAAGSYM**A**PSTVLQQIDSVAD	Yes	SYM**A**PSTVL	Yes	9	<0.01 (SB)
Nlrp1a	ADQNPELCLHWSQ**P**QPVHTLLGSLLGK	No	Q**P**QPVHTL	No	488	0.43 (SB)
Vmn2r67	TCYNLYNAIYAV**P**HSLHEMLLQQIDTW	Yes	V**P**HSLHEML	Yes	649	<0.01 (SB)
Fbxo31	LPPHDPHVGDPMR**L**KPLFRIHLMERKA	No	DPMR**L**KPLF	No	9028	4.80
Oas1h	FRSVSFKLSYPKF**P**QEVEFHMQTAYDA	Yes	F**P**QEVEFHM	No	16	0.02 (SB)
Cyp3a59	TKKILKFKIFDPF**R**LSTILFPFLTPIY	Yes	FDPF**R**LSTI	No	946	0.06 (SB)
F2r	VNPRSFFLRNPSE**S**TFELVPLGDEEEE	No	NPSE**S**TFEL	No	111	0.09 (SB)
Ube3a	VMENSNLHSPEYL**Q**MALPLFCKAMCKL	Yes	EYL**Q**MALPL	No	45	0.06 (SB)
Vps13b	DPEEVSSLEEYIP**S**RHTSVTLLKCTCT	Yes	EYIP**S**RHTSV	Yes	68	0.10 (SB)
Esd	WKANYRMYSYVTE**G**LPQLINANFPVDP	No	SYVTE**G**LPQLI	No	309	0.46 (SB)
Otud7b	SGGEAFAPIPFGG**L**YLPLEVPPNRCHC	No	IPFGG**L**YL	No	273	0.26 (SB)
Gzmg	GGHEVKPHSRPYM**T**FIKSVDIEGKKKY	No	KPHSRPYM**T**F	No	148	0.21 (SB)
Gm9733	TAKIIVASLLGPK**R**LLVIVSVMYRHKK	Yes	LGPK**R**LLVI	Yes	85	0.01 (SB)
Letmd1	PKADVKNLISYVV**I**KTRAINGSYHRFL	Yes	SYVV**I**KTRA	Yes	114	0.16 (SB)
4933425L06Rik	FQYVFEKVEILSY**V**EEDSYIKAIVRDF	Yes	SY**V**EEDSYI	No	52	0.07 (SB)
Wnk1	SEYPYSECQNAAQ**V**YRRVTSGVKPASF	Yes	**V**YRRVTSGV	No	470	0.69 (WB)
Dcaf4	SQAKGWKATQIFH**E**SAVTSVQVFKEEQ	Yes	FH**E**SAVTSV	No	107	0.16 (SB)
Ctsm	TGRESDGRKYWLV**R**NSMGTQWGNKGYM	Yes	KYWLV**R**NSM	No	36	0.05 (SB)
Exosc5	GSLLACCLNAACM**P**LVDAGVPMRALFC	No	M**P**LVDAGVPM	No	1613	1.30
Sult1e1	FSEFVEKFMQGQV**S**YGSWYDHVKAWWE	No	**S**YGSWYDHV	No	337	0.51 (WB)
Cpxm2	EMTTTDDLDFKHH**K**YKEMRQLMKVVNE	Yes	**K**YKEMRQLM	Yes	137	0.20 (SB)
Sf3b2	EEEIDRTPWGELE**L**SDEESSEEEEEEE	No	TPWGELE**L**	No	11050	5.80
Plch1	FQEADTDENQGTL**P**FEEFCVFYKMMSL	No	L**P**FEEFCVF	No	91	0.07 (SB)

Gene	SLP		pMHC-I		IC50(nM)	%EL rank
	Sequence	Immunogenic	Sequence	Immunogenic		
Impdh2	KHLSSQNRYFSEA**N**KIKVAQGVSGAVQ	Yes	RYFSEA**N**KI	No	105	0.16 (SB)
Coch	SLEEFSVFGNIVY**V**SVSSICGAAVHRG	No	VY**V**SVSSI	No	74	0.11 (SB)
Slc44a2	TSARNAFFLLMRN**T**IRVAVLDKVTDFL	Yes	FFLLMRN**T**I	Yes	29	0.04 (SB)

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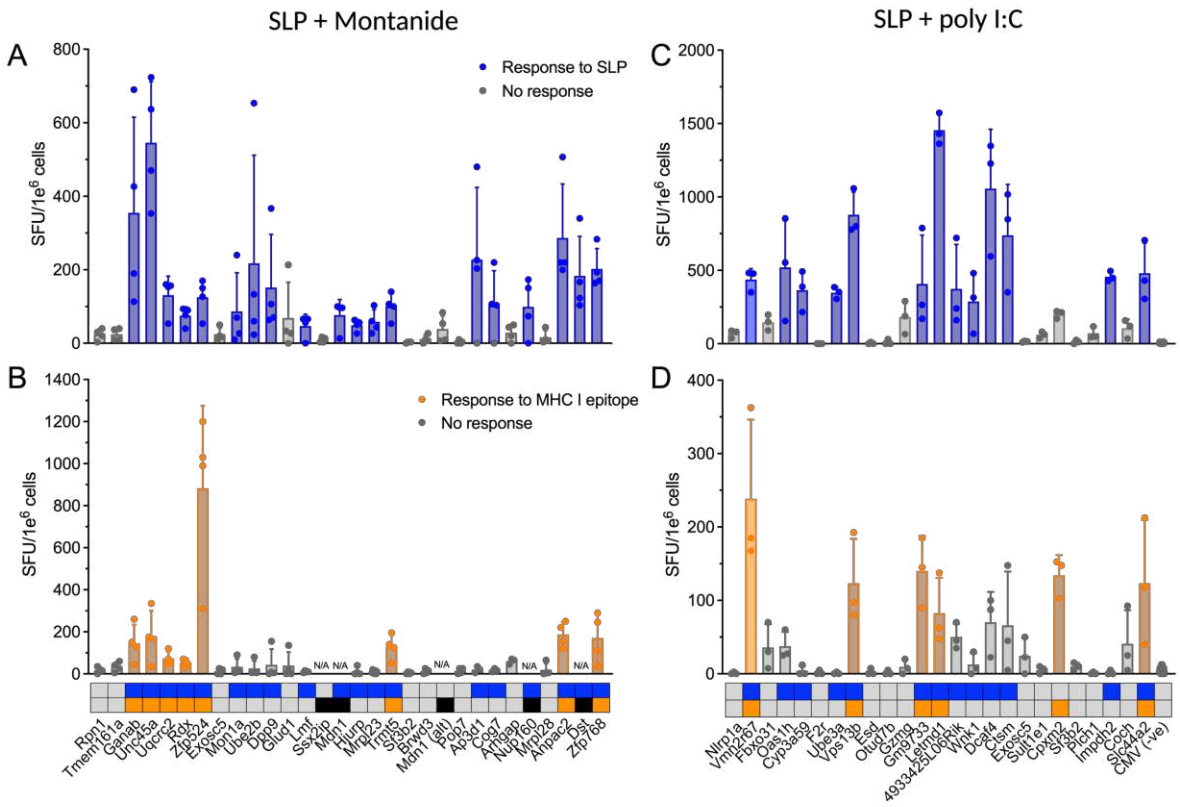


Figure 2. Predicted neoantigens generate *de novo* T-cell responses following vaccination. Non-tumour-bearing mice were vaccinated with pools of 5 SLPs corresponding to genomically predicted neoantigens, adjuvanted with either (A-B) Montanide or (C-D) poly I:C. T-cell IFNγ responses were assessed to the immunising SLPs (A & C) and the corresponding predicted MHC-I epitope (B & D) where possible. A positive response (indicated in blue for SLP; orange for MHC-I epitope) was determined by One-Way ANOVA with Dunnett's correction comparing multiple neoantigen peptides to negative control peptide. Data are presented as mean ±SD spot forming units per million cells; n= 3-4 mice per group.

Vaccines that either boost spontaneous responses or induce *de novo* T-cell responses are not protective

Because very few studies screen for spontaneous T-cell responses, we sought to determine if vaccines containing spontaneously immunogenic neoantigens were more effective than those consisting entirely of induced neoantigens. Mice were vaccinated with the complete repertoire of spontaneous neoantigens identified in the AB1-HA model, including Unc45a, Uqcrc2, Elp2

and Vmn2r67. Vaccine efficacy was compared to that obtained with four of the induced neoantigens from the following genes: tRNA methyltransferase 5 (Trmt5); zinc finger protein 524; (Zfp524); alpha glucosidase 2 alpha neutral subunit (Ganab); and midasin AAA ATPase 1 (Mdn1). Trmt5, Zfp524, and Ganab were selected because they elicited T-cell responses to both the SLP and MHC-I epitope (Figure 2A and B). Mdn1 was selected because it elicited a response to the SLP and encoded 6 strong and 3 weak potential MHC-I binders. Notably, Mdn1 had two mutations, only one of which was immunogenic and used in vaccine protection experiments (Figure 2A). Neoantigen peptides were adjuvanted with Montanide or poly I:C and delivered subcutaneously over three injections (Figure 3A). Neither spontaneous nor induced neoantigens were protective when administered with Montanide (Figure 3B and C). Induced neoantigens adjuvanted with poly I:C offered a minor protection and survival benefit (Figure 3D and E); however, this was not reproducible. Failure to control tumour development was not due to vaccination failure, as T-cell responses were detected in the spleens of vaccinated mice (Figure 3F-I).

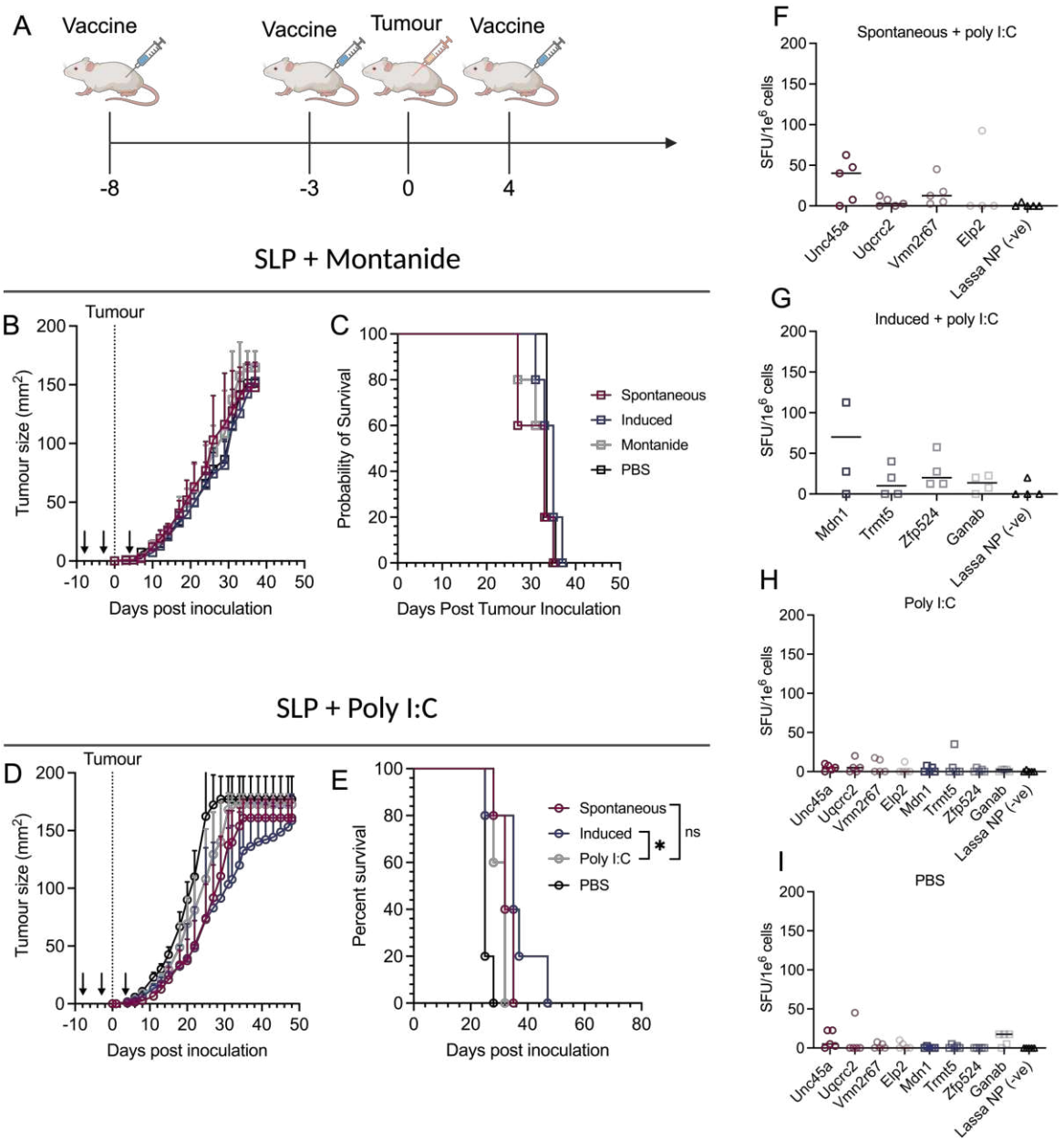
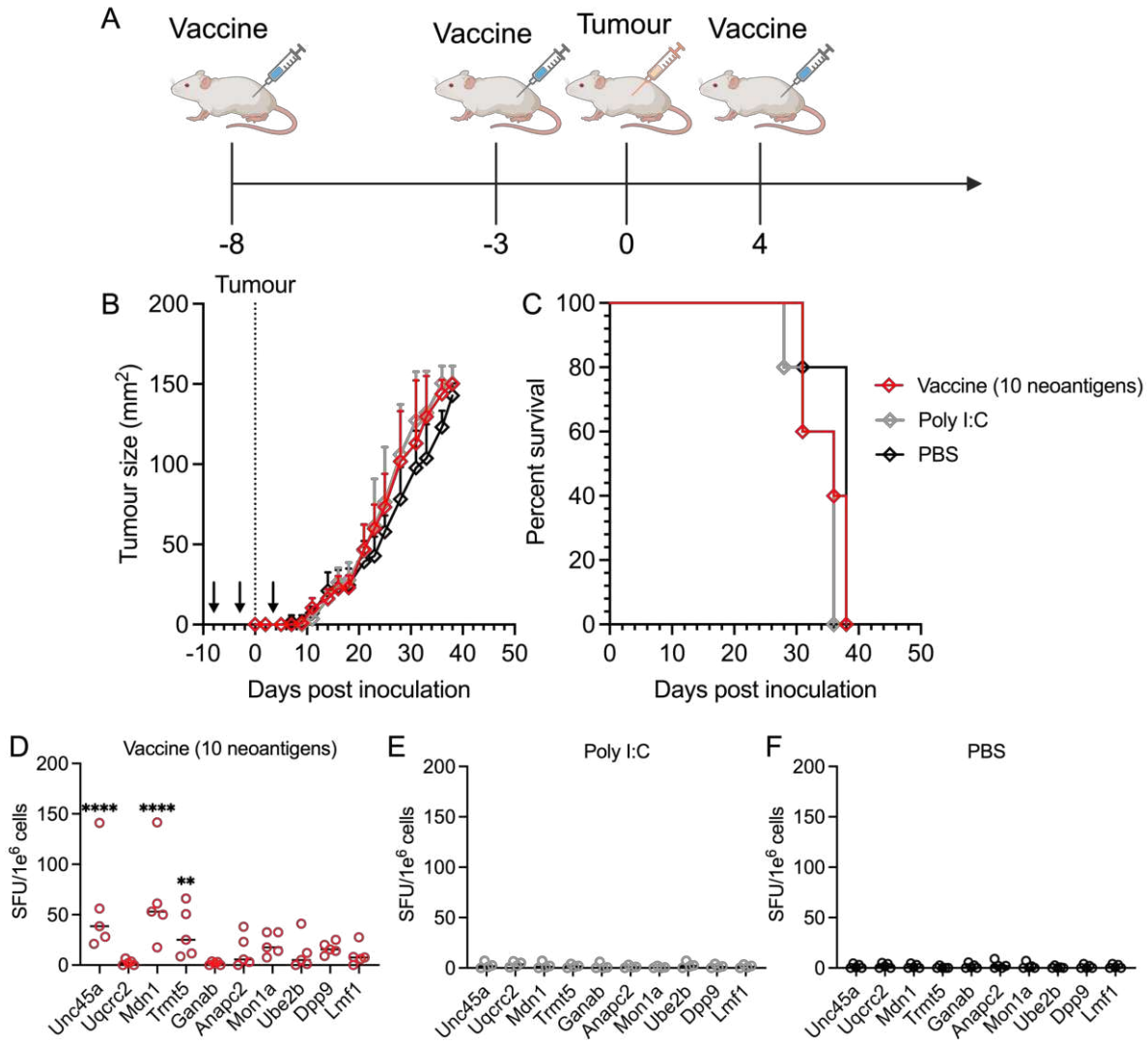


Figure 3. **Vaccination with either spontaneous or induced neoantigens is not protective**. **(A)** Mice were vaccinated with SLP for four spontaneous or four induced neoantigens, plus an adjuvant; either Montanide or poly I:C. Mice received a total of three doses of a vaccine, two doses prior to tumour challenge and one after AB1-HA inoculation. Control mice were vaccinated with either Montanide, poly I:C, or PBS. **(B)** Tumour growth curves and **(C)** survival curve from mice vaccinated with Montanide adjuvanted vaccines. **(D)** Tumour growth curves and **(E)** survival curve from mice vaccinated with poly I:C adjuvanted vaccines. **(F)** Splenic T-cell IFNγ responses to vaccinating peptides in spontaneous antigen vaccinated mice. **(G)** Splenic IFNγ T-cell responses to vaccinating peptides in induced antigen vaccinated mice.

(H-I) Splenic IFNγ T-cell responses to all spontaneous and induced neoantigens in **(H)** adjuvant (Poly I:C) or **(I)** PBS injected mice; n=5 mice per group. One-Way ANOVA with Dunnett's correction was used to compare multiple neoantigen peptides to a negative control peptide. Kaplan-Meier method was used to compare survival of each group to adjuvant controls (Montanide or poly I:C). *$p<0.05$.

Increasing the number of vaccine neoantigens does not enhance efficacy

Given that clinical trials typically target 10-20 neoantigens in vaccines to overcome low rates of immunogenicity[7-10,28-30], we reasoned that too few antigens were included in our vaccine for tumour control. As such, we tested if protection could be achieved by vaccinating with 10 bona fide neoantigens (Figure 4A). This included a mix of spontaneous and induced neoantigens, including Unc45a, Uqcrc2, Mdn1, Ganab, and Trmt5, as well as five additional induced neoantigens from the following genes: anaphase-promoting complex subunit 2 (Anapc2); MON1 vesicular trafficking associated A (Mon1a); ubiquitin-conjugating enzyme E2B (Ube2b); dipeptidyl peptidase 9 (Dpp9); and lipase maturation factor 1 (Lmf1). However, vaccination with 10 neoantigens failed to protect mice from tumour challenge (Figure 4B and C). Failure to control tumour development was again not due to vaccination failure, as strong T-cell responses to 3/10 antigens were detected in the spleens of vaccinated mice (Figure 4D). Vaccinating with the same 10 neoantigens emulsified in Montanide also failed to protect and was associated with considerably weaker antigen-specific T-cell responses (Supplementary Figure 1). These data demonstrate that simply increasing the number of neoantigens in vaccines does not improve vaccine efficacy.



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208 **Figure 4. Increasing the number of vaccine neoantigens does not control tumour growth.**
209 **(A)** Mice received a total of three doses of a vaccine containing SLP corresponding to ten
210 immunogenic neoantigens, plus poly I:C. Control mice received poly I:C alone or PBS. Mice
211 received two vaccine doses prior to tumour challenge and one dose after AB1-HA inoculation.
212 **(B)** Mean +SD tumour growth. **(C)** Survival curve. **(D-F)** T-cell IFNγ responses to neoantigens
213 in the spleen of **(D)** vaccinated mice, **(E)** mice treated with Poly I:C alone, or **(F)** mice treated
214 with PBS alone. Data are from n=5 mice per group. Two-Way ANOVA with Dunnett's
215 correction was used to compare the response to each peptide across groups. Kaplan-Meier
216 method was used to compare survival of each group to poly I:C. **p<0.01, ****p<0.0001.

A dominant antigen, which on its own is insufficient for tumour control, improves neoantigen vaccine efficacy

We hypothesised that the addition of a dominant antigen may enhance vaccine efficacy. To address this, we utilised the model antigen, influenza haemagglutinin (HA), which is stably expressed under antibiotic selection by the AB1-HA cell line[22,23]. Mice were vaccinated with SLP for two spontaneous neoantigens (Unc45a and Uqcrc2) and two induced neoantigens (Trmt5 and Mdn1), plus the minimal MHC-I HA epitope or its corresponding SLP (pentameric vaccine; Figure 5). Inclusion of the minimal HA epitope in the pentameric vaccine protected 2/10 mice and extended survival compared with poly I:C alone (Figure 5A and G). Given that the minimal HA sequence contained 45% hydrophobic residues, and hydrophobic peptides may form immunogenic self-aggregates[31], we also confirmed that immunogenicity was not simply due to peptide aggregation using a scrambled HA sequence (Supplementary Figure 2A-D). By contrast, inclusion of the corresponding HA SLP protected 5/15 mice completely, and a sixth animal until day 60, conferring a significant survival benefit over poly I:C alone (Figure 5B and G). This was accompanied by a strong T-cell response to the HA SLP and MHC-I epitope, a suppressed response to the Uqcrc2 and Trmt5 vaccine SLPs, and an enhanced response to the Vmn2r67 non-vaccine SLP (Supplementary Figure 2E and F). Vaccines that excluded HA and contained only the four neoantigens (tetrameric vaccine) provided no protection from tumour challenge, and immunisation with the HA SLP alone protected only 1/5 mice (Figure 5C and D). Together these data suggest that a dominant antigen such as HA is necessary but on its own not sufficient for neoantigen vaccine efficacy.

Having demonstrated that addition of a dominant antigen improved vaccine efficacy, we revisited the relative protective capacity of either spontaneous or induced neoantigens when combined with HA. As seen previously, vaccines containing only spontaneous or inducible

neoantigens failed to prevent tumour development (Supplementary Figure 3C and E) despite driving some, albeit low, neoantigen-specific T-cell responses (Supplementary Figure 3J and L). The addition of HA to spontaneous neoantigens protected 1/5 mice and delayed tumour growth, however this was comparable to vaccination with HA alone and did not confer a survival benefit (Supplementary Figure 3A, B, and F). Similarly, HA combined with induced neoantigens delayed tumour growth but did not lead to improved survival (Supplementary Figure 3A and D). These data indicate that a dominant antigen such as HA improves vaccine efficacy but, at least within the limitations of this model, only when a combination of induced and spontaneous neoantigens are included.

For several mice that received pentameric vaccines, tumour growth was suppressed for some time prior to rapid outgrowth (Figure 5A and B). We reasoned that, in these tumours, escape of immunological control was a result of antigen loss. To address this, tumour lines were developed from 6 different "escaped" tumours following vaccines that contained both mutation-derived neoantigens and HA (T1-6 indicated in Figure 5A and B). To determine if each retained the capacity to express HA, cell lines were stained with the HA specific monoclonal antibody HA18. In all six cases, HA expression was reduced or absent relative to the parental AB1-HA cell line, and all cell lines had lost resistance to G418 selection in culture (Figure 5H and I). For four of the six *ex vivo* tumour cell lines, this was also accompanied by loss of HA mRNA expression, as determined by RT-PCR (Figure 5J). RT-PCR for each of the neoantigens included in the pentameric vaccine was also performed, and sequencing of PCR products revealed that expression of neoantigen-specific mutations was maintained by all *ex vivo* cell lines (data not shown). These data indicate that loss of the dominant antigen, HA, was sufficient for tumour escape.

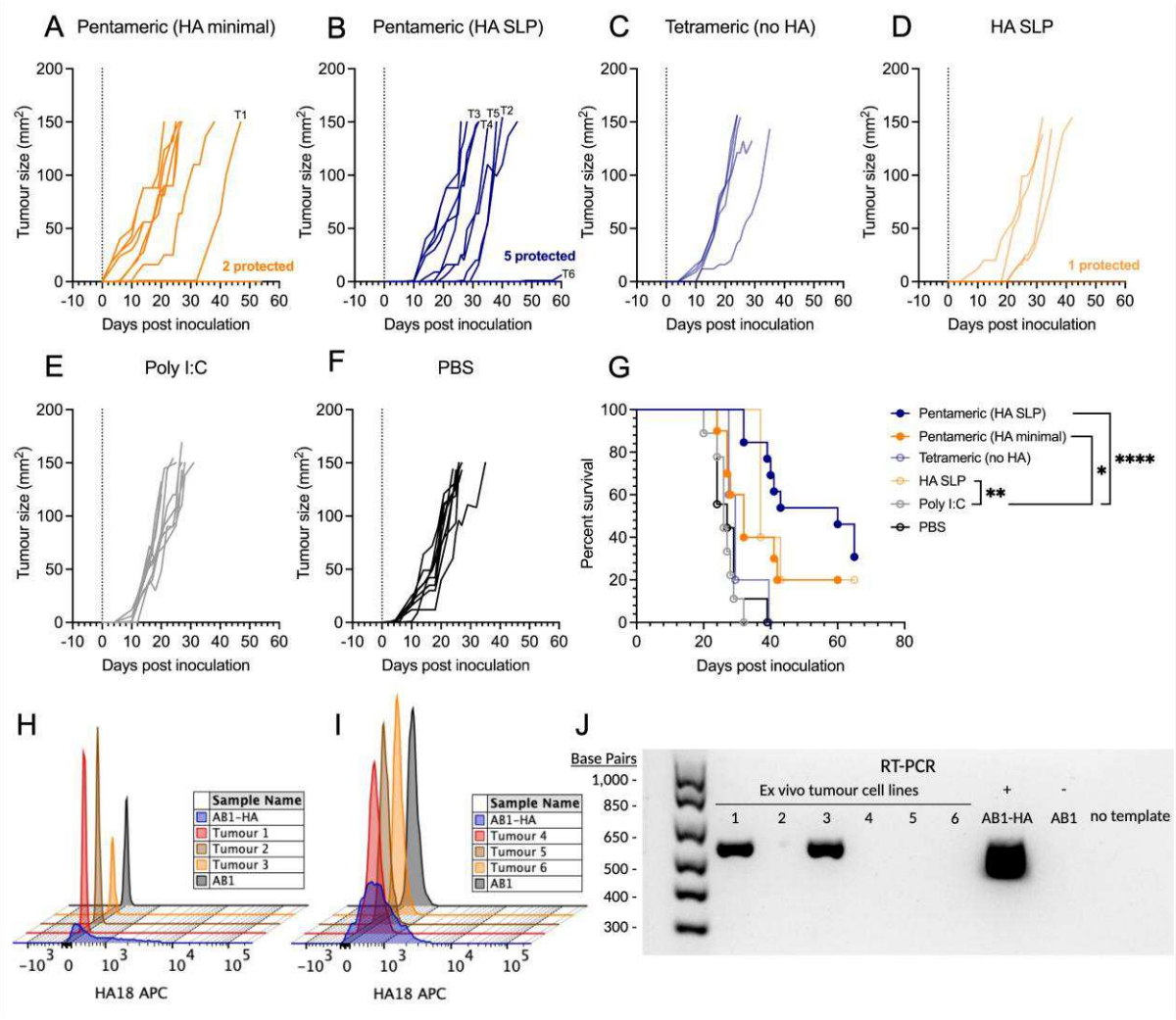


Figure 5. HA is required for vaccine-induced protection and is lost when tumours escape vaccine-mediated control. Mice received three doses of SLP + poly I:C vaccines subcutaneously by the base of the tail, on days -8, -3, and 4 relative to tumour inoculation as indicated by arrows. On day 0, mice were inoculated on the flank with subcutaneous AB1-HA tumours, and mice were euthanised when tumours reached 130-150mm^2. Select tumours (numbered T1-T6) were cultured ex vivo and assessed for antigen loss. **(A-F)** Individual tumour growth curves and **(G)** Survival curve for vaccines +/- HA, HA only, or controls. **(H-J)** Expression of HA by ex vivo vaccine-escaped tumours. Cell lines were established from tumours (T1-T6 from **(A)** and **(B)**) that grew out following a period of vaccine-mediated control. Cell lines were assessed for **(H-I)** surface HA expression by flow cytometry and **(J)** HA mRNA expression by RT-PCR compared to AB1 (negative control) and AB1-HA (positive control); n=5-15 mice per group. Kaplan-Meier method was used to compare survival of each group to poly I:C. *p<0.05, **p<0.01, ****p<0.0001.

Taken together, this study demonstrated that a strong, dominant antigen is necessary but on its own insufficient for vaccine-induced protection. Protective vaccines also required a combination of spontaneous and inducible neoantigen-specific T-cell responses. Importantly however, losing only HA expression following vaccination was enough for tumours to escape immunological control, suggesting a central role of this dominant antigen in maintaining strong anti-tumour immunity.

Discussion

Neoantigen vaccines have recently gained traction with promising early results in clinical trials[2,7-12], however low TMB cancers such as mesothelioma have largely been excluded from investigation thus far. Given a much more limited range of neoantigens to choose from for such tumours, it is essential to understand what features of neoantigens are required for vaccine efficacy. We show here, in a mouse model of mesothelioma, that vaccines comprised of either spontaneously recognised neoantigens, neoantigens that induce *de novo* T-cell responses, or a mix of both did not confer any significant tumour protection. Further, simply increasing the number of vaccine candidates was insufficient to provide protection. However, a mix of spontaneous and induced neoantigens combined with a strong antigen was sufficient for tumour protection. Tumour escape was associated with loss of this dominant antigen, but not the mutation derived neoantigens.

To our knowledge the relative efficacy of neoantigen vaccines containing spontaneous versus induced neoantigens has not been explored by other groups. Some groups posit that pre-screening is important and ensures immunogenicity of 100% of vaccine epitopes[14-17,32-34]. While this poses a substantial logistical challenge, if spontaneously recognised neoantigens are more protective than induced neoantigens, there is considerable incentive to pre-screen as part of the antigen selection process. Others reason that pre-existing T-cell responses are already suppressed[35,36], and/or that tumour cells expressing these antigens are likely to have already been immunologically edited[5], suggesting that the induction of *de novo* responses is more important for tumour control. In the present study we found no evidence that spontaneously recognised neoantigens were more protective than neoantigens that induced *de novo* responses following vaccination. This was not due to AB1-HA being poorly immunogenic; spontaneous and vaccine-inducible immunogenicity rates were 3.8% and 58% respectively, consistent with

311 those reported across a range of tumour types. Notably, the two strongest spontaneously
312 recognised mutation-derived targets, Unc45a and Uqcrc2, were also among the most promising
313 candidates based on *in silico* prediction and would likely have been selected in a personalised
314 vaccine approach regardless of pre-screening[7-9,28,29,37-39]. These findings therefore provide little
315 support, within this model, for routine pre-screening of spontaneous T-cell responses to vaccine
316 candidate neoantigens.
317
318 An alternative strategy for overcoming uncertainty in antigen selection is to increase the
319 number of vaccine targets, thereby increasing the likelihood that sufficient relevant antigens
320 are included and reducing the opportunity for escape mediated by antigen loss. Most vaccine
321 clinical trials include 10-20 computationally predicted neoantigens, with 40-60% reportedly
322 immunogenic post-vaccination[7-9,28,29]. However, in our model, increasing the vaccine from four
323 to ten experimentally validated, mutation-derived neoantigens did not improve tumour
324 protection, despite eliciting measurable T-cell responses. Thus, antigen number and the ability
325 to induce a measurable T-cell response were themselves insufficient to establish vaccine
326 efficacy. This is consistent with data from other models, where only a subset of immunogenic
327 neoantigens were capable of protection[4]. Qualitative features of individual tumour antigens,
328 such as mutation position[37,40,41] and abundance on tumour MHC-I[37,40], rather than simply the
329 breadth of the vaccine response, may be critical.
330
331 In the present study protection was observed only when HA was added to the mutation-derived
332 antigen vaccine. Although HA is a strong viral antigen, it was originally introduced into AB1
333 to promote tumour rejection and was insufficient to consistently do so[22,23]. This raises the
334 possibility that effective multi-antigen vaccination may depend on the presence of both
335 dominant and subdominant antigens. Antigen hierarchies can be established in tumours,

influencing the magnitude and breadth of T-cell responses and subsequent tumour clearance[42,43]. In some mouse models, dominant neoantigens can suppress subdominant responses and limit tumour control[42,43]. In others, stronger neoantigens enhance responses to weaker targets when clonally co-expressed[44]. In our model, the inclusion of HA appeared to suppress responses to two of the other vaccine antigens, Uqcrc2 and Trmt5. Notably, Uqcrc2 was computationally predicted to bind the same MHC-I (H-2K^{d}) more strongly than HA, highlighting that predicted binding affinity alone does not determine dominance. Other factors like tumour antigen heterogeneity may also influence how antigen-specific responses compete or cooperate, though this was not explored here. Despite their reduced magnitude, T-cell responses to subdominant neoantigens may still have provided broader tumour recognition and constrained escape, evidenced by the lack of consistent protection with HA alone. Consistent with strong selective pressure against dominant antigens, tumours that escaped vaccine-mediated control lost HA expression, while the mutation-derived neoantigens were retained. Importantly, tumour escape following antigen loss has also been demonstrated with mutation-derived targets, indicating that this is not unique to strong viral antigens[5]. Loss of a dominant antigen may provide an opportunity to selectively boost responses to retained subdominant neoantigens, though whether this could restore tumour control remains to be determined.

Together, our results demonstrate that spontaneous T-cell recognition, neoantigen number, and measurable immunogenicity alone do not determine protection in this model. The requirement of a dominant antigen highlights that there may be hierarchical relationships between antigens that need to be considered in vaccine design and scheduling. Further, neoantigen selection is only one obstacle to successful cancer vaccination, with other challenges not addressed here including widespread[45] and local[46] immunological perturbation, T-cell exhaustion[3], and a refractory tumour vasculature that limits immune cell infiltration[47]. Vaccine efficacy is also

361 strongly influenced by adjuvant and delivery platform, including emerging mRNA and lipid
362 nanoparticle approaches, and cancer vaccines will almost certainly be combined with ICI[2,48].
363 Nonetheless, this study has provided evidence of neoantigen features that may influence the
364 vaccine candidate selection process, and demonstrated proof-of-principle for neoantigen
365 vaccination in mesothelioma and other low TMB cancers.

Materials and methods

Mice

Eight- to ten-week-old female BALB/c were purchased from the Animal Resource Centre, Murdoch, Australia and maintained under standard specific pathogen free housing conditions at the Harry Perkins Institute of Medical Research (HPIMR). All animal procedures were approved by the HPIMR Animal Ethics Committee and performed according to the Australian code for the care and use of animals for scientific purposes.

Cell culture and tumour inoculation

The murine mesothelioma cell line AB1-HA was generated previously[22] and expresses the Puerto Rico/8/34/Mount Sinai hemagglutinin (HA). Cells were maintained in R10 media; RPMI 1640 (ThermoFisher Scientific, VIC, Australia) supplemented with 20 mM HEPES, 0.05 mM 2-mercaptoethanol, 100 units/mL penicillin (CSL, VIC, Australia), 50 μg/mL gentamicin (David Bull Labs, VIC, Australia), 10% foetal calf serum (FCS; Invitrogen, VIC, Australia), and 50 mg/mL of geneticin (G418; Life Technologies). Cells were passaged when 70-80% confluent, were used below 30 passages, and routinely tested negative for Mycoplasma contamination by PCR. Mice were inoculated subcutaneously (s.c.) into the flank with 5×10^5 AB1-HA cells suspended in 100 μl of PBS using one 26-gauge needle per injection. Length and width tumour measurements were monitored every 2-3 days using callipers to calculate tumour area (mm^2).

Neoantigen prediction and selection

Previous whole exome sequencing (WES) and RNA sequencing (RNAseq) data from AB1 and AB1-HA were re-analysed using a previously established genomics-based bioinformatic pipeline[24]. WES data were aligned to the mm10 murine reference genome (UCSC mm10/NCBI

GRCm38) using the Burrows-Wheeler Aligner[49], and RNAseq data were aligned to mm10 using Hisat2[50]. Mutation calling was performed with reference to normal samples obtained from the lung or tail of BALB/c mice. Somatic non-synonymous single nucleotide variants (nsSNVs) and insertions or deletions (indels) were called using Mutect2[51], Varscan v2.3[52], and Somatic Sniper v1.0.5[53]. The called variants were subjected to a filtering step using the default settings and a variant calling file (vcf) was created based on a "PASS" for these filtering parameters. A total of 1576 mutations were called from AB1/AB1-HA.

Neoantigen prediction was performed using Antigen Garnish[26]. MHC binding of peptides (nmers) of 8-15 AA around the mutation point was calculated and the "consensus score" IC50 value (with 95% CI) of four predictors netMHC, netMHCpan, MHCflurry and MHCnuggets was generated for each nmer. The DAI for each nmer was also available from the Antigen Garnish output. Post processing was performed to generate a 31 mer "core peptide" associated with each nmer by selecting the 15 amino acids around the mutation index point to facilitate peptide selection. Peptides were prioritised based on eluted ligand (EL) rank above 0.5%, IC50 of less than 500 nM, or a DAI above 2. Long peptides (17-29 amino acids) that corresponded to a highly ranked short peptide (8-13 amino acids) were also prioritised for screening.

Synthetic peptides

For screening purposes, crude (30% purity) peptides comprising 143 short (8-14mer) and 20 long peptides (17-29mer) were obtained from Mimotopes (Mulgrave, Victoria) and resuspended in 100% DMSO (Sigma-Aldrich) at 2-4 mg/mL. For ELISpot assays peptides were pooled (5-12 peptides per pool) and used at 1 ug/ml/peptide. For vaccine purposes, 27 mer peptides were synthesised by Mimotopes (Mulgrave, Victoria) at >90% purity and resuspended in 25% DMSO at 5-10 mg/mL. For all neoantigen vaccine peptides the mutation

was positioned at the 13 residue in the peptide. To overcome the high degree of hydrophobicity of the HA sequence, the minimal MHC-I epitope is offset within the SLP (KVDGVKLESMGIYQILAIYSTVASSLV). All peptides included in vaccine protection experiments are listed in Supplementary Table 2.

Neoantigen vaccination

Mice were vaccinated with 20 ug of each peptide (up to 100 ug for vaccines containing 5 peptides) in a total volume of 100 uL. Vaccines were adjuvanted with either Montanide ISA^{TM} 51 (emulsified 1:1 with peptide) (Seppic, Paris, France) or 100 ug of VacciGrade^{TM} high molecular weight polyinosinic-polycytidylic acid (poly I:C) (InVivoGen, California, USA). For vaccine protection experiments, three doses were administered at days -8, -3, and day 4 relative to tumour-inoculation (day 0). Mice were euthanised when tumours reached 130-150 mm^2 or as dictated by ethical constraints. At indicated time points, ipsilateral axillary and inguinal tumour-draining lymph nodes (LN) and spleens were collected for *ex vivo* T-cell assays. For epitope validation and screening of inducible neoantigen responses, non-tumour-bearing mice received vaccinations on days 0, 5, and 12, with *ex vivo* analyses conducted on day 19.

IFNγ ELISpot

T-cell responses to neoantigens were assessed by IFNγ Enzyme Linked ImmunoSpot (ELISpot) assay as per the manufacturer's instructions and as previously described[54]. Briefly, MAIPS4510 Multiscreen® plates (Millipore, Billerica, MA) were coated with anti-mouse IFNγ antibody overnight (clone AN18; Mabtech, Victoria), and blocked with R10 media. Single-cell suspensions of tumour-draining LN or spleens were plated at 1×10^5 or 2×10^5 cells/well respectively, and were stimulated with 1 μg/mL of individual peptides or peptide

pools overnight at 37°C. Assay controls were Dynabeads® Mouse T-activator CD3/CD28 beads (positive; Thermo Fisher Scientific, NSW, Australia), cells and DMSO peptide diluent (background), and irrelevant viral peptide controls (negative; Lassa virus nuclear peptide, or CMV pp89). Plates were subsequently developed with biotinylated anti-mouse IFNγ antibody (clone R4-6A2, Mabtech), conjugated horseradish-peroxidase (Mabtech), and tetramethylbenzidine (TMB) substrate (Mabtech). Plates were air dried completely and spots were enumerated using the Autoimmun Diagnostika AID ELISpot reader (Advanced Imaging Devices GmbH, Strassberg, Germany) with software version 7.x. All ELISpot assays were performed in duplicate and results presented as IFNγ Spot Forming Units (SFU) per 10^6 cells. For each mouse the mean background number of SFU/10^6 cells was subtracted from the value for test wells.

Magnetic activated cell sorting

To compare the $CD4^+$ vs $CD8^+$ response to Elp2, T-cells were purified from spleens of Elp2-vaccinated mice by magnetic activated cell sorting (MACS) prior to ELISpot. Cells were purified using the $CD4^+$ T-Cell Isolation Kit, mouse (Miltenyi Biotec, Rhineland, Germany), $CD8a^+$ T-Cell Isolation Kit, mouse (Miltenyi Biotec), and MidiMACS™ Separator (Miltenyi Biotec) according to the manufacturer's instructions. Cells were plated at 5 x 10^4 per well with 5 x 10^3 naïve BALB/c spleen cells as APCs.

RT-PCR

Ex vivo tumours were harvested, washed in RNA later (Qiagen), cut in pieces of maximum 20 mg, and immediately flash-frozen in liquid nitrogen and stored at -80 °C until nucleic acid extraction. DNA and RNA extraction was performed by using All Prep DNA/RNA Mini Kit from QIAGEN, according to the manufacturer's instructions. Neoantigen loss was assessed by

PCR analysis of cDNA to measure mRNA expression. PCR primers were HA forward: TTGCTATGGCTGACGGAGAAGG, HA reverse: CTGGATTGAATGGACGGAGTGTTC (539 bp product); Trmt5 forward: GTTGACAAAAACCCAGGA, Trmt5 reverse: TAGAGTGTAGCAGTGGGA (743 bp product); Unc45a forward: ACCCCAAGGACAAACCAA, Unc45a reverse: GAGGGATACGACTGCAGA (702 bp product); Uqcrc2 forward: GTGGAGGTGAAATTAGAGAG, Uqcrc2 reverse: GACTTTGATGTGCTTGCT (652 bp product); Mdn1 forward: AGGAGGAAGCTGATGTAA, Mdn1 reverse: GAGACAATCCTGGACGAA (762 bp product) (Bioneer, Daejeon, Korea).

Flow cytometry

2 x 10^5 cells per well were stained with eFluor™ 780 Fixable Viability dye (Invitrogen) diluted at 1/4,000 in PBS + 1 mM EDTA for 20 min in the dark at room temperature (RT). To measure surface expression of HA, cells were incubated for 30 min at RT with HA18-APC diluted 1/25 in FACS staining buffer (FSB); PBS, 2% FCS (Invitrogen), and 1 mM EDTA. The HA-specific HA18 antibody was provided by Kathleen Davern (HPIMR, WA, Australia) and labelled in-house using a Lightning-Link® APC Conjugation Kit as per the manufacturer's instructions and stored at 4°C up to 7 days prior to staining (Abcam, Cambridge, UK). Cells were washed twice and resuspended in FSB. Data were acquired using a BD LSRFortessa™ (BD Biosciences, New Jersey, USA) with 50,000 live events collected per sample where possible, and analysis was completed using FlowJo™ Software version 10 (BD Biosciences).

Statistics

Tumour growth was analysed by mixed model ANOVA or by the SPSS MIXED procedure. Kaplan Meier method was used for survival analysis with Log-rank test (Mantel-Cox) to

491 analyse significance. ELISpot data were analysed using Ordinary One-Way or Two-Way
492 ANOVA with Dunnett's correction for multiple comparisons. Data are presented as individual
493 values with mean ±SD unless otherwise stated. All analyses were performed using IBM SPSS
494 statistics version 22 (IBM SPSS, Chicago IL) and GraphPad Prism V11 (GraphPad Software,
495 California, USA).

Data availability

The data and code that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions

J.B., S.M., W.S.Y., N.M., B.W.S.R., J.C., and A.J.R. were responsible for conception and experimental design. J.B., S.M., W.S.Y., G.H., P.C., S.C.N., E.J.R., and N.M. conducted experiments and data collection. J.B. and I.M.D. performed data analysis. B.W.S.R., J.C., and A.J.R. supervised the study. J.B., C.C., F.X.R., J.C., and A.J.R. wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

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